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Production of linalool, cis- and trans-nerolidol, and trans,trans-farnesol by Saccharomyces fermentati growing as a film on simulated wine

by

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Die Bildung von Linalool, cis- und trans-Nerolidol sowie trans,trans-Farnesol durch eine Decke von Saccharomyces fermentati auf einem Modellwein

Zusammenfassung. — Die deckenbildende Form von *Saccharomyces fermentati* erzeugte im Verlauf von 10 Wochen auf einem simulierten Fino-Sherry, der als einzige flüchtige Kohlenstoffverbindung Äthanol enthielt, geringe Mengen von Linalool, cis-Nerolidol, trans-Nerolidol und trans,trans-Farnesol. Es ist zwar nicht bekannt, ob *Saccharomyces cerevisiae* bei der Vergärung von Traubensaft ebenfalls Terpene bildet; trotzdem scheint Vorsicht geboten zu sein, wenn *Vitis-vinifera*-Sorten aufgrund der in den Weinen gefundenen Terpene charakterisiert werden.

Introduction

The odors of wines — the aroma and bouquet component — are of great interest to enologists because they are, together with the taste and color, of importance in distinguishing among the many types and styles of wines. Odors in wines result from the presence in the wine of chemical compounds at concentrations greater than the levels required for sensory perception. These compounds can come from the grape, passing through the fermentation and processing steps without alteration, from the activity of the yeast in producing volatiles from the non-volatiles of the grape or in altering certain of the grape volatiles to produce different volatile compounds, from volatiles extracted from the wood during the aging phase and, finally, from redox interactions among the various volatiles during the course of bottle aging. Drawert and co-workers (1966) stress the importance of the rapid chemical changes that can occur when the berry is first crushed, and Suomalainen and Nykänen (1964) have repeatedly emphasized the importance of the contribution to odor made by the yeast in fermenting the sugar and other non-volatiles of the grape. Banthorpe *et al.* (1972), in an elegant review of the biosynthesis of monoterpenes, summarize the meager information on biosynthesis of terpenes by yeasts. The route to cholesterol and squalene in yeasts, starting with acetate, goes through mevalonate to geraniol and nerol, thence to higher molecular weight products. Details of the conversions are uncertain in several areas. Lanza and Palmer (1977) have shown that with the fungus, *Ceratocystis moniliformis*, growing in aerobic shake culture in 3 % potato-dextrose broth, geraniol and a number of other monoterpenes are produced from acetate, mevalonic acid and L-leucine, and that the terpenes are excreted into the medium in the free form. The yeast, *Kluyveromyces*

lactis, which is fairly closely related to the *Saccharomyces cerevisiae* of wine fermentations, in aerobic shake culture produced free citronellol, linalool, and geraniol according to the research of DRAWERT and BARTON (1978).

RAPP and HASTRICH (1976) and SCHREIER and co-workers (1976), in an extensive series of experiments, report that wines from different grape varieties can be differentiated on the basis of the terpene compounds coming from the grape used in making the wine. They were unable to differentiate these same wines on the basis of compounds known to be produced by the yeast during fermentation.

We report now the finding that *Saccharomyces fermentati*, a wine yeast, growing as a film on a simulated wine under microaerophyllic conditions, produces low concentrations of some mono- and sesquiterpene alcohols.

Materials and methods

Simulated wine culture medium

22 l of culture medium designed to resemble film sherry but having ethanol as the only carbon containing volatile were prepared. 19 l of distilled water were distributed among four 6-l Erlenmeyer flasks. To one flask 295 g of Difco Bacto #0392-15 yeast nitrogen base and 22 g of yeast autolysate (Charles Pfizer and Company, Inc., #723G) were added. 65 g of reagent grade potassium acid tartrate were distributed between two of the remaining flasks. The four flasks were stoppered with cheesecloth and autoclaved at 120 °C and 15 psi pressure for 15 min. To the cooled sterile solutions combined in a 12-gal Pyrex carboy were added 3-l of 95 % ethyl alcohol and 19 ml of 6 n HCl. Each l of culture medium thus contained 130 ml of ethanol, 2.95 g of potassium acid tartrate, 13.4 g of yeast nitrogen base, and 1 g of yeast autolysate. The added HCl reduced the pH to 3.4. All steps in the preparation of the culture were designed to maintain sterility. The carboy, closed with multiple layers of cheesecloth, was placed in a room maintained at a constant temperature of 20 °C.

Yeast

An inoculum of *Saccharomyces fermentati* growing in the film form on Palomino wine was transferred from the barrel in the solera room to the surface of the culture medium in the glass carboy. The minimum possible amount of liquid was transferred. By the end of the 4th week a thick complete film had formed on the surface of the simulated wine.

Controls

22 l each of two control media were prepared. These control media were identical to the experimental medium described above except that to the first no yeast was added and to the second only cells that had been killed by exposure to 70 % ethanol with vigorous shaking for 5 min. No living cells could be found in a sample of this killed yeast.

Isolation and concentration of yeast metabolites

10 weeks after inoculation of the culture media with yeast, each was separated from the cells by carefully syphoning the clear liquid from beneath the film. Final

quantities of liquid were recovered from the yeast by suction filtration. Yeast metabolites were extracted from the liquid medium in a 30-l stainless steel rotating drum extractor with CH_2Cl_2. Four portions of the solvent were used over a period of 192 h to yield 600 ml of extract. Acids were removed from the extract by washing 4 times with 30 ml portions of ice-cold 5 % aqueous $NaHCO_3$ solution. The acid-free extract was washed once with saturated NaCl solution and dried overnight over anhydrous NA_2SO_4. After removal of the drying agent by filtration, most of the CH_2Cl_2 was removed in a rotary evaporation at 0 °C and approximately 10 mm Hg pressure. The two control media samples were also allowed to stand for a 10-week period and were worked up exactly as described above.

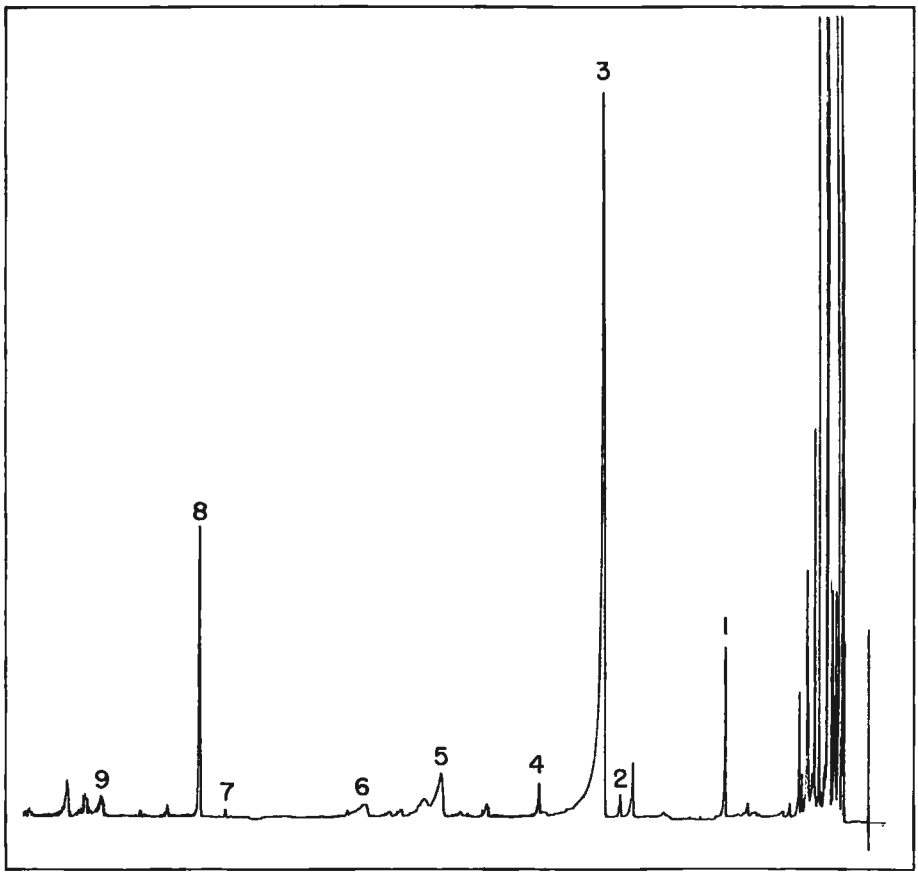

Fig. 1: Gas chromatographic trace of yeast culture volatiles on glass capillary coated with SE-54. Peak 1 = γ-butyrolactone, 2 = linalool, 3 = 2-phenylethanol, 4 = diethyl succinate, 5 = 4-hydroxy-5-ketohexanoic acid-γ-lactone, 6 = 4,5,dihydroxyhexanoic acid-γ-lactone, 7 = *trans*-nerolidol, 8 = *cis*-nerolidol, and 9 = *trans,trans*-farnesol.

Gaschromatogramm der Aromaverbindungen aus der Hefekultur; Glaskapillare: SE-54. Peak Nr. 1 = γ-Butyrolacton, 2 = Linalool, 3 = 2-Phenyläthanol, 4 = Bernsteinsäure-diäthylester, 5 = 4-Hydroxy-5-ketocapronsäure-γ-lacton, 6 = 4,5-Dihydroxycapronsäure-γ-lacton, 7 = *trans*-Nerolidol, 8 = *cis*-Nerolidol, 9 = *trans,trans*-Farnesol.

Separation and identification of yeast metabolites

The concentrated essence fractions were separated into components and analyzed with an F & M Scientific (Hewlett-Packard) model 810 gas chromatograph with FID and fitted with a glass capillary column 0.25 mm i.d. by 35 m coated with SE-54 silicone. The 1-μl samples injected were split so that 1/40 of the sample entered the analytical column. Injector temperature was 230 °C and column temperature was 70 °C for 15 min followed by a 1 °/min temperature rise program to 250 °C. The helium carrier gas was adjusted to a flow rate of 17 cm/s. Hydrogen, air, and helium make-up gas flows were 30, 300, and 60 ml/min. A Varian model CDS 111 data system was used to measure retention times and relative peak areas.

Retention indices of compounds were calculated from corrected retention times relative to corrected retention times of a homologous series of straight chain aliphatic hydrocarbons.

GC-MS separations and identifications were accomplished using a Finnigan model 9500 gas chromatograph coupled to a Finnigan model 3200 quadrapole mass spectrometer. A Finnigan model 6000-1 MS digital interface to a Finnigan model 6000 data system fitted with a Zeta Research series 100 plotter permitted storage, manipulation and plotting of the mass spectra of the separated components.

Results and discussion

Fig. 1 shows a gas chromatographic trace of the volatiles from the simulated wine which had had the culture of *S. fermentati* growing as a film completely covering the surface of wine in the container for 10 weeks. While the yeast protein-building byproducts, 2-phenylethanol (3), gamma-butyrolactone (1), and diethyl succinate (4) are clearly present, the most significant feature is the presence of trace amounts of linalool (2), *trans*-nerolidol (7), *trans,trans*-farnesol (9) and a larger amount of *cis*-nerolidol (8). The table lists the retention indices of the compounds isolated from the yeast culture medium. It is evident that, with the possible exception of linalool, the identities of the compounds are well supported by the retention indices. Fig. 2 presents the mass spectrum of linalool determined on the Finnigan quadrapole mass spectrometer. The agreement of this sample spectrum with that of an authentic

Retention indices of compounds isolated from yeast culture medium
Retentionswerte der aus dem Hefekulturmedium isolierten Verbindungen

Compound	Retention indices — unknown (known) Carb. 20M		SE-54	
γ-Butyrolactone	1638	(1632)	948	(950)
Linalool	1562	(1552)		
2-Phenylethanol	1888	(1887)	1147	(1146)
Diethyl succinate	1684	(1680)	1200	(1199)
4-Hydroxy-5-ketohexanoic acid-γ-lactone			1300	(1300)
trans-Nerolidol	1991	(1991)	1547	(1547)
cis-Nerolidol	2029	(2027)	1574	(1576)
trans,trans-Farnesol	2370	(2363)	1741	(1740)

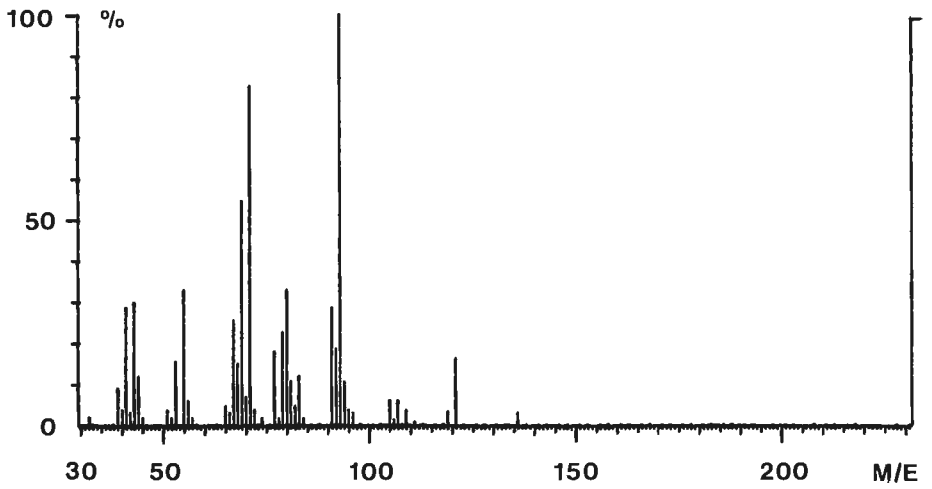

Fig. 2: Mass spectrum of linalool from yeast culture medium.
Massenspektrum von Linalool aus dem Hefekulturmedium.

reference run on the same instrument and with literature spectra is excellent. There can be no question that small quantities of linalool were present in the yeast culture medium.

Mass spectra of *trans*-nerolidol, *cis*-nerolidol and *trans,trans*-farnesol of the yeast culture extracts obtained with the Finnigan quadrapole mass spectrometer, are essentially identical with those of known materials run under the same conditions on the same instrument and match very closely with literature spectra.

Gas chromatographic traces of extracts from the control medium to which no yeast had been added and the control medium to which 70 %-ethanol-killed yeast had been added did not show peaks for the four terpenes found in the medium which had supported yeast growth. The evidence is thus very strong that *S. fermentati* growing as a film for 10 weeks on a defined medium simulating flor sherry produces linalool, *trans*-nerolidol, *cis*-nerolidol, and *trans,trans*-farnesol.

In comparing our research with that previously done with yeasts and terpenes there are some significant differences. In much of the earlier work, cell-free extracts of yeast were used in tracing the steps in the biosynthetic pathway from mevalonic acid to the terpenes. In most cases where intact cells were used, they were employed in aerated and agitated culture media. In no case that we can find, has a typical wine yeast been used, and certainly not under conditions typical of a normal wine fermentation. The research currently being reported was conducted with *S. fermentati,* the normal wine yeast employed in the Jerez de la Frontera district of Spain. While this organism is a vigorous fermenter of hexose sugars and is used for this stage of wine production in Jerez, we used the yeast in its film forming stage in which ethanol serves as the carbon source for growth. When in the form of a thick film on the surface of a wine or simulated wine, the cells in the upper part of the film are under aerobic conditions, but those at the lower surface of the film in contact with the wine are under strong reducing conditions. Reducing conditions more nearly resemble the situation existing in the active fermentation of hexose sugars to make wine. It is not known if the partial pressure of oxygen in contact

with the yeast cell has any influence on the terpene production, but the fact that terpenes were secreted into the liquid medium suggests that the terpenes are produced by the cells at the lower interface of the yeast film and thus under conditions of very low partial pressures of oxygen.

Classification of plants based on their chemical composition is a very old idea and one that has recently become of increasing interest because of the sensitivity and precision of modern gas chromatographic-mass spectrometric analytical data gathering techniques. Red *Vitis vinifera* grapes have been separable from red American species grapes on the basis of the fact that the former contain anthocyanin monoglucosides while the latter contain diglucosides as their pigments (Ribéreau-Gayon 1954). Within the red *V. vinifera* cultivars, Pinot noir is distinguishable from the others on the basis of its lack of acylated anthocyanins (Rankine *et al.* 1958). Fruit of the *V. labruscana* species is separable from that of the *V. vinifera* cultivars on the basis of its content of methyl anthranilate, while within the species *V. vinifera* the cultivars Muscat and Riesling are differentiated from most of the others by their relatively high concentrations of monoterpenes, in particular, linalool. Indeed, within the smaller group of white *V. vinifera* cultivars typically grown in Germany, Rapp and Hastrich (1978) have shown that the ratios among the various monoterpenes can be used to distinguish not only one cultivar from another but also region and season of growth. Precise and detailed analyses of the various grapes, combined with computer program methods of multiple discriminant analysis hold great promise for differentiating among the many cultivars and perhaps regions and seasons of growth for many other species and regions.

Since wine is much easier to store and transport than ripe grapes, it would be highly desirable to be able to differentiate among grape cultivars and regions and seasons of growth on the basis of analyses of the wines produced from the respective lots of grapes. Among the several attempts to do this using gas chromatographic-mass spectrometric analytical data, that of Schreier *et al.* (1976) is typical. As these authors note, success depends upon selecting compounds identified as coming from the grape rather than being produced by the action of the yeast on the non-volatiles of the crushed grapes. Thus, differentiation among a number of German white grape cultivars was based on the patterns of concentrations of a number of monoterpenes present in these cultivars. The research currently being reported is of significance in that it documents the production of terpenes by wine yeasts. It is to be noted that the small quantities of terpenes produced resulted from 10 weeks metabolic activity of the film yeast. A fino sherry of 5—7 years average age might well be expected to contain significantly higher concentrations. It is to be noted, also, that production of terpenes by *S. fermentati* in the film form supports only the presumption that *S. cerevisiae* in normal alcoholic fermentation would also do so. However, caution should be used in trying to differentiate grape cultivars based on wine analyses.

Summary

Linalool, *cis*-nerolidol, *trans*-nerolidol, and *trans,trans*-farnesol are produced in low concentrations by the wine yeast *Saccharomyces fermentati* growing as a film for 10 weeks on the surface of a simulated fino sherry containing ethanol as the only volatile carbon containing compound. It is not known whether *Saccharomyces cerevisiae* also produces terpenes while fermenting grape juice to wine. However,

caution seems advisable in differentiating among *Vitis vinifera* cultivars on the basis of terpenes found in the wines.

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